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Domingues, MM, Macrae, FL orcid.org/0000-0002-7092-0422, Duval, C orcid.org/0000-0002-4870-6542 et al. (7 more authors) (2016) Thrombin and fibrinogen γ' impact clot structure by marked effects on intrafibrillar structure and protofibril packing. Blood, 127 (4). pp. 487-495. ISSN 0006-4971

https://doi.org/10.1182/blood-2015-06-652214

Thrombin and fibrinogen γ' impact clot structure by marked effects on intrafibrillar structure and protofibril packing

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Supplementary Online Material

Methods

Normal pooled plasma

Free-flowing blood was obtained from the antecubital vein of 23 healthy volunteers. The blood was centrifuged within 30 minutes at 2,400g for 20 minutes to separate the plasma. The plasma samples were pooled, aliquoted and stored at -80˚C. Informed written consent was obtained from each volunteer in accordance with the declaration of Helsinki. Ethical approval was obtained from the University of Leeds Medical School Ethical Committee.

Purification of human plasminogen-depleted fibrinogen

In order to remove traces of FXIII and other plasma proteins, human plasminogen-depleted fibrinogen (Merck Millipore, Darmstadt, Germany) was resuspended in Tris-buffered saline (TBS; 50 mM Tris-HCl, 100 mM NaCl, pH 7.4) and further purified through IF-1 antibody (Kamiya Biomedical Company, Seattle, WA) affinity

chromatography, as described by Takebe *et al.*[1] and Smith *et al.*[2] using an AKTAavant 25 purification system (GE Healthcare, Little Chalfont, UK). Briefly, fibrinogen was applied to the IF-1 column in the presence of 10 mM $CaCl_2$, sequentially washed with Tris-buffers containing 1 M and 0.3 M NaCl, and eluted in TBS containing 5 mM of ethylenediaminetetraacetic acid (EDTA). The purified fibrinogen was dialysed in TBS, purity analysed using a NuPAGE unit (Invitrogen; Paisley, UK) with 4-12% Bis-Tris gradient gel (Invitrogen; Paisley, UK), and aliquots were stored at -80°C. The human plasminogen-depleted IF-1 purified fibrinogen was composed of 91% γA/γA and 9% γA/γ' fibrinogen as determined by specific ELISA (see below).

Purification of γA/γA and γA/γ' fibrinogen

γA/γA and γA/γ' fibrinogens were isolated from IF-1 purified human plasminogen-depleted fibrinogen using anionic exchange chromatography as previously described by Wolfenstein-Todel and Mosesson[3] and Cooper *et al.*[4] In brief, the variants were separated using a DE-52 column on the AKTAavant 25. Before injection, fibrinogen was dissolved in 39 mM Tris, 65 mM H_3PO_4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 5 mM ε-aminocaproic acid, pH 8.6. Samples were eluted using a concave gradient from 0% to 100% over 13 column volumes (with increments of 5% over the first 6 and increments of 10% over the next 7 column volumes) of 500 mM Tris, 650 mM H_3PO_4, 0.5 mM PMSF, 1 mM benzamidine and 5 mM ε-aminocaproic acid, pH 4.2. Purity of the γA/γA and γA/γ' preparations was checked on a NuPAGE unit with 4-12% Bis-Tris gradient gels, and aliquots were stored at -80°C. Fibrinogen concentration was determined by absorbency ($E^{280\ nm}_{1\ mg.mL^{-1}} = 1.51$).[5]

ELISA for fibrinogen γ'

Fibrinogen γ' concentrations were measured by ELISA based on the method described by Uitte de Willige *et al*.[6] Nunc MaxiSorp 96 well plates (Thermo Scientific, Waltham, MA) were coated with 2.G2.H9 monoclonal antibody (Santa Cruz Biotech, CA, 200µg/mL, final concentration) in 50 mM sodium carbonate pH 9.5 overnight at 4°C. Plates were blocked for 1 hour at room temperature in washing buffer (1% BSA, 50 mM Triethanolamine, 100 mM NaCl, 10 mM EDTA, 0.1% Tween-20, pH 7.5). Plasma was diluted 1/1600 and 1/3200 in dilution buffer (50 mM triethanolamine, 100 mM NaCl, 10 mM EDTA, 0.1% Tween-20, 10 mM benzamidine, pH 7.5) and incubated in the plate for 1 hour at room temperature. An HRP conjugated goat polyclonal antibody against human fibrinogen (1/20.000 in dilution buffer) (Abcam, Cambridge, MA) was added and incubated for 1 hour at room temperature. 100 µL 3,3',5,5'-Tetramethylbenzidine substrate buffer was added and the reaction was stopped after 10 minutes with 50 µL 1M H_2SO_4. The plate was read at 450 nm at 37°C in a Thermo Fisher Multiscan Go Microplate reader (Thermo Fisher Scientific, Waltham, MA). After blocking and between each incubation step, the plate was washed three times with washing buffer. Fibrinogen γ' antigen levels were measured at two dilutions in duplicate, against a standard curve of purified fibrinogen γA/γ'.

Transmission Electron Microscopy

Clots were prepared from mixtures that contained 1 $mg.mL^{-1}$ fibrinogen, 2.5 mM $CaCl_2$ and 0.1, 1.0 and 10 $U.mL^{-1}$ human α-thrombin (Sigma-Aldrich; St Louis, MO). Samples were prepared on the surface of a carbon coated copper grid (AGAR Scientific; Stansted, UK), washed (3x) with TBS, after which 1% uranyl acetate in

water was added for 5 minutes as contrast agent, followed by removal of excess water with filter paper, and drying of the samples in air. The samples were analysed with a JEOL 1400 TEM (Tokyo, Japan) transmission electron microscope (120 kV).

Scanning Electron Microscopy

Clots for scanning electron microscopy (SEM) were generated by adding 100 µL of γA/γA and γA/γ' fibrinogen (1 $mg.mL^{-1}$, final concentration) to 10 µL of activation mixture (human thrombin, 0.1 $U.mL^{-1}$ and $CaCl_2$, 10 mM, final concentrations, in TBS). The clotting mixture was immediately transferred to open permeation tubes. Clots were left to form in a humidified chamber at room temperature for 2 hours. The tubes containing the clots were connected via plastic tubing to a reservoir containing TBS. Clots were left to wash in TBS for 2 hours, followed by washing in sodium cacodylate for another 2 hours. The clots were then fixed for 1 hour by washing with 2% glutaraldehyde, after which they were removed from the permeation tubing into a pierced Eppendorf. The clots inside the pierced Eppendorfs were left overnight in 2% glutaraldehyde. Clots were further washed three times in sodium cacodylate (67 mM $C_2H_6AsNaO_2$, pH 7.4) and dehydrated in a series of increasing acetone concentrations (30–100%). Clots were critical-point dried with CO_2, mounted onto stubs and sputter-coated with platinum using a Cressington 208 HR (Cressington Scientific Instruments, Watford, UK). Each clot was imaged in five areas at 50,000x magnification using a next generation Cold Field Emission SEM (Hitachi SU8230 FESEM, Schaumburg, IL).

Turbidimetric analysis

The average fiber diameter was determined from the wavelength dependent turbidity of the fibrin clot as described by Carr and Hermans[7] and more recently by Yeromonahos et al.[8] The wavelength dependent absorbance of fully formed fibrin gels prepared from fibrinogen at different thrombin concentrations is shown in Figure S1A. The absorbance of the gels decreased with increasing thrombin concentrations. We applied Yeromonahos's updated model to determine the fiber diameter from the turbidity at different thrombin concentrations using the wavelength dependent turbidity at λ>500 nm.

Optical density values, *D*, obtained from the scan were transformed into turbidity values, τ, using the following relationship[8]:

$$\tau = 1 - e^{(-D.l.ln(10))}$$

where l is the sample thickness in cm.

For a solution of randomly orientated fibers of long and thin rods smaller than the incident wavelength beam, the turbidity is[7,8]:

$$\tau\lambda^5 = \frac{2\pi^3 C n_s \mu}{N}\left(\frac{dn}{dc}\right)^2 \frac{44}{15}\left(\lambda^2 - \frac{184}{154}\pi^2 a^2 n_s^2\right)$$

where N is Avogadro's number, λ is the incident wavelength (cm), μ is the fiber protein mass/length (Da.cm^{-1}) and a is the fiber radius (cm), C the initial fibrinogen mass concentration (g.mL^{-1}), n_s=1.33 is the solvent refractive index, and dn/dc=0.17594 cm^3/g is the solution refractive index change with protein concentration.[9]

The average number of protofibrils/cross section of fibrin fiber, *Np*, and the average radius, a, of the fibrin fibers were determined by plotting $\tau\lambda^5$ as a function of λ^2 which produced a straight line where the mass/length ratio (μ) was calculated from the slope.[8] The ordinate at the origin allowed the calculation of the square of the average radius. For all the experimental conditions used, plots of $\tau\lambda^5$ versus λ^2 provided straight line fits (Figure S1B) with accurate correlation coefficients (R^2>0.98).

The average number of protofibrils per fibre cross section was determined from μ by:

$$N_p = \frac{\mu}{\mu_0}$$

where μ_0=1.44x10^{11} Da/cm is the mass/length ratio of a single protofibril, determined previously by Carr and Hermans.[7] In order to further describe the packing of the protofibrils inside the fibre we next calculated the distance of the protofibrils. Fibres of radius a and protein mass density, δ, are made by n protofibrils packed at an average distance z from each other:

$$z = \sqrt{\frac{\mu_0}{\delta}}$$

Assuming a random packing fraction changes the result only by a few percent, the average protein density in the fibers (δ) is:

$$\delta = \frac{\mu}{(\pi a^2)}$$

Thromboelastometry

A ROTEM delta (Hartlepool, UK) device was used for thromboelastometric analysis. Citrated blood was obtained from one healthy donor after informed consent. Ethical approval was obtained from the University of Leeds Medical School Ethical

Committee. Whole blood, normal pooled plasma (see above), and deficient plasma supplemented with γA/γA and γA/γ' (300 µL) were incubated at 37°C in a heated cup. For studies using supplemented deficient plasma, both fibrinogen-deficient plasma and γA/γA and γA/γ' fibrinogen were concentrated in Vivaspin 500 of 3,000 kDa cutoff and Vivaspin 20 100,000 kDa cutoff (Generon, Maidenhead, UK), respectively, to achieve a 2x concentrated deficient plasma and a γA/γA and γA/γ' fibrinogen preparation of 6 $mg.mL^{-1}$. After that, plasma and fibrinogen were mixed to achieve a 1x plasma and a final γA/γA and γA/γ' fibrinogen concentration of 3 $mg.mL^{-1}$. Human α-thrombin (final concentrations of 1.0 and 10 U.mL-1) and $CaCl_2$ (final concentration of 10 mM) were added (final volume 340 µL) to start clot formation. Immediately after the addition of thrombin and $CaCl_2$, the pin was suspended within the cup and the analysis started. The pin oscillates to the left and right inside the cup by rotating through an angle of 4.75°. The movement is initiated from the pin. As fibrin forms between the cup and pin the impedance of the rotation of the pin is detected and a trace of the maximum clot firmness (MCF) is generated over time. We collected extended data on clot formation for over 1 hour and measured the final MCF for each thrombin concentration in triplicate.

Statistical analysis

Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical analysis was performed using Unpaired t-test and Dunnett's test in one-way analysis of variance (ANOVA) (GraphPad v6.05; La Jolla, USA), except for the comparison of γA/γA versus γA/γ' in both purified and supplemented deficient plasma, for which a Bonferroni's test in two-way analysis of variance (ANOVA) was used. P-values <0.05 were considered to indicate statistical significance.

Figures and Tables

Table S1 – Viscoelastic properties of fibrin clots produced with human plasminogen-depleted IF-1 purified fibrinogen in the absence of calcium chloride analyzed with magnetic tweezers.

[Thrombin], U.mL^{-1}	G', Pa	G'', Pa	$tan\ \delta$
0.1	0.534±0.006	0.189±0.036	0.353±0.068
1.0	0.355±0.060	0.106±0.004*	0.297±0.051
5.0	0.294±0.051*	0.099±0.050*	0.299±0.167

Data represented as mean ± SD, n=3. Statistical significance are denoted with * $p<0.05$ for comparison between 0.1 U.mL^{-1} and the remaining thrombin concentrations.

Table S2 – Quantitative values of the fiber radius, protofibril number, protofibril density and protofibril distance obtained by turbidimetry performed on the clots with purified γA/γA, γA/γ', γA/γA:γA/γ' 60%:40% and γA/γA:γA/γ' 91%:9%. Data represented as mean ± SD, n=3.

	Fiber Radius, nm		**Protofibril number**		**Protofibril density, g.cm^{-3}**		**Protofibril distance, nm**	
Thrombin, U.mL^{-1}	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0
γA/γA	83.6±2.9	72.9±1.8	256.5±21.6	95.8±4.5	0.266±0.009	0.137±0.002	9.5±0.2	13.2±0.1
γA/γ'	80.8±0.5	77.9±0.9	196.7±3.5	103.0±0.6	0.230±0.003	0.129±0.002	10.2±0.1	13.6±0.1
γA/γA:γA/γ' (60%:40%)	80.9±0.9	66.0±5.2	208.3±9.6	80.1±8.3	0.242±0.003	0.141±0.008	9.9±0.1	13.1±0.4
γA/γA:γA/γ' (91%:9%)	81.3±1.6	67.4±6.6	211.5±11.4	79.9±9.4	0.244±0.004	0.134±0.011	9.9±0.1	13.4±0.5

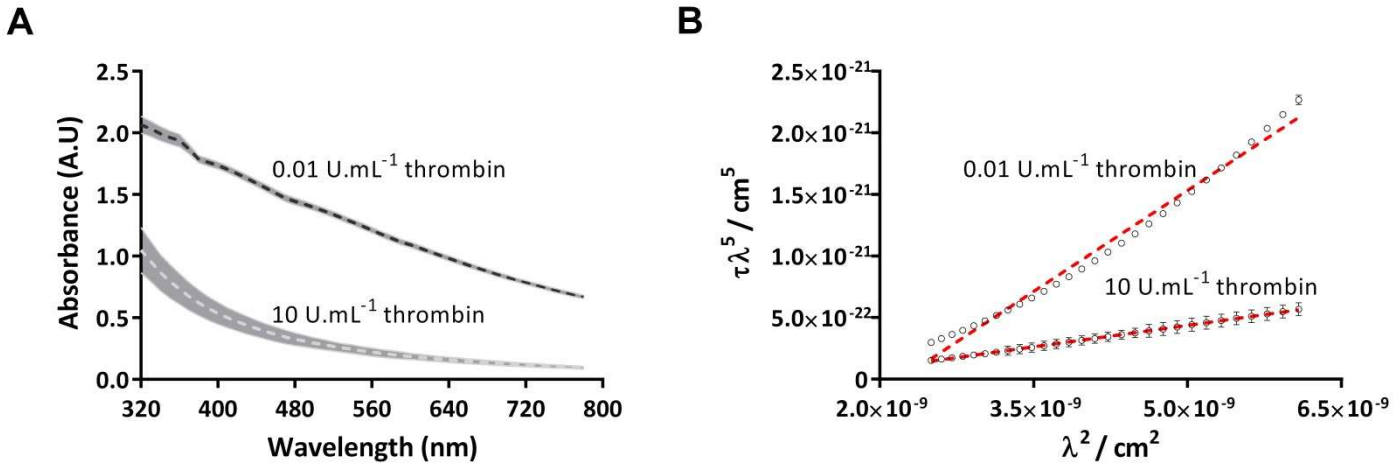

Figure S1: Determination of the fiber radius and protofibril number from turbidimetric measurements. A) UV-Vis spectra in the fully formed fibrin clot (example shown from the human plasminogen-depleted IF-1 purified fibrin clot) prepared with 0.01 and 10 $U.mL^{-1}$ of thrombin. B) Determination of radius and mass-length ratio from the turbidity linear fits.

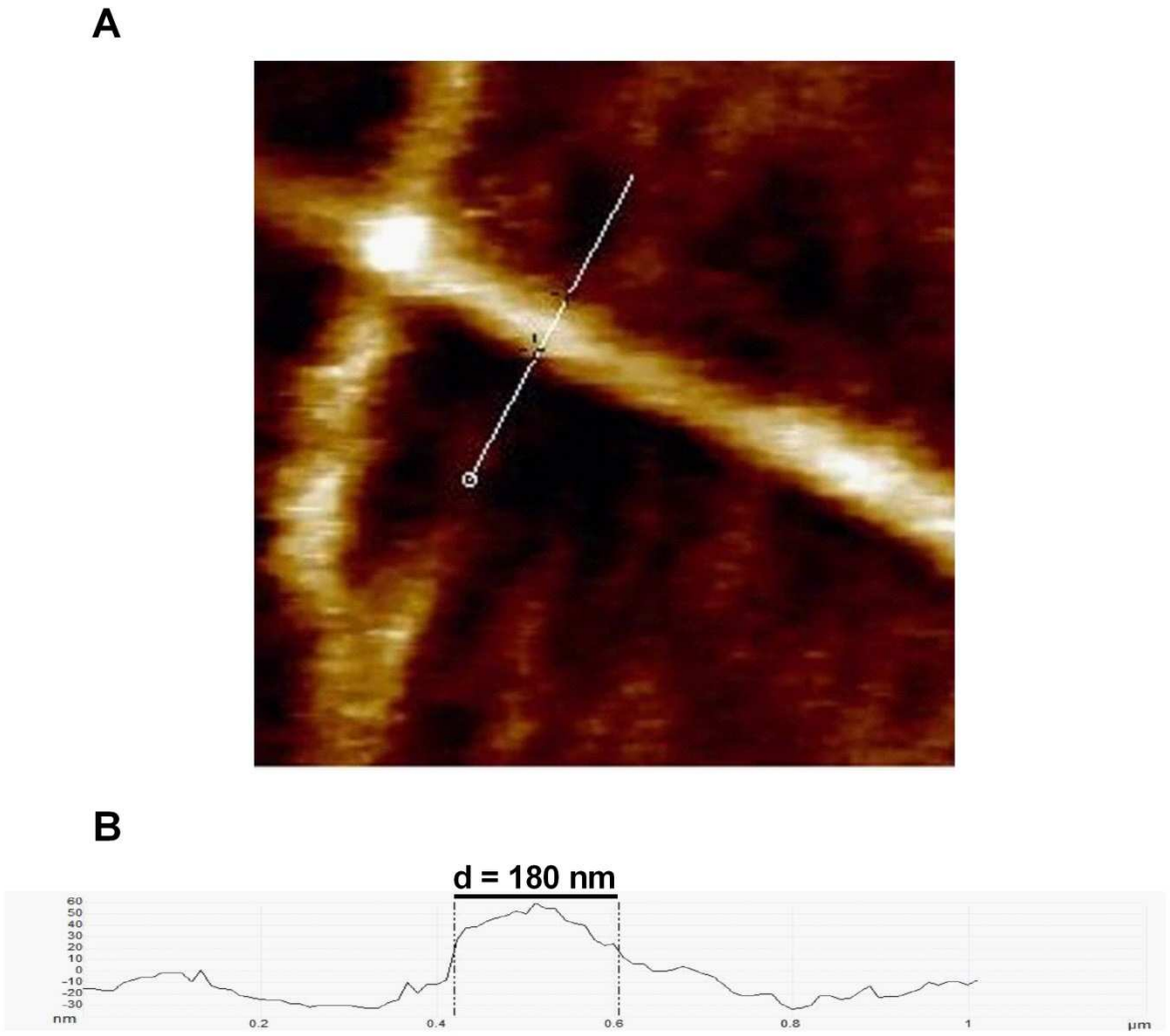

Figure S2: Determination of fibrin fiber radius from atomic force microscope images. A) Cross-section of the fibrin fiber produced with 1 $U.mL^{-1}$ thrombin. B) Determination of the diameter (for this cross-section the diameter is 180 nm) of the fibrin fiber at half maximal height.

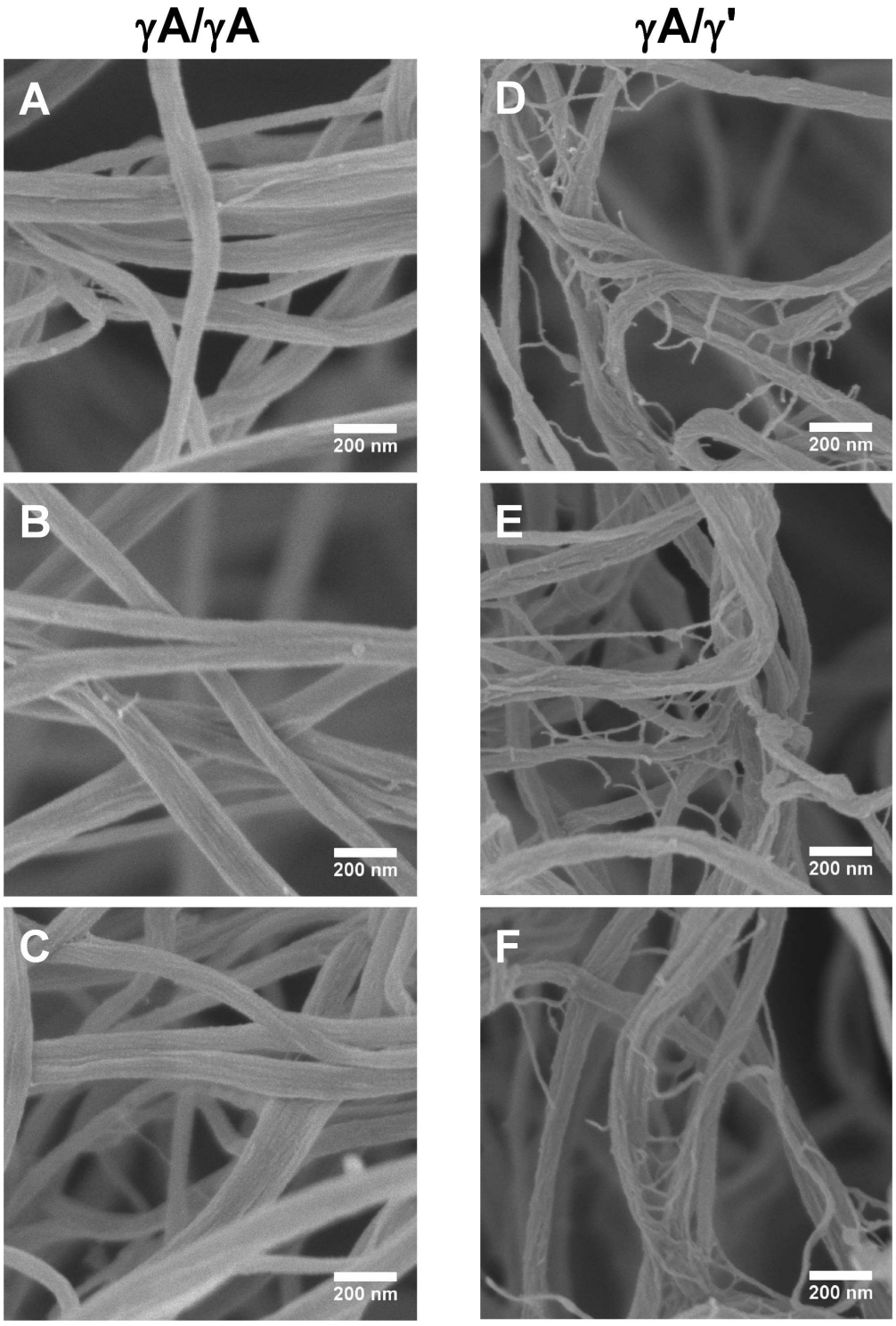

Figure S3: SEM images of fibrin fibers formed with γA/γA fibrinogen and γA/γ' fibrinogen. A to C) γA/γA; D to F) γA/γ' fibrin fibers. Fibrin clots were produced with 1 mg.mL^{-1} fibrinogen, 0.1 U.mL^{-1} thrombin and 10 mM $CaCl_2$ and imaged with a Cold Field Emission SEM as detailed in the supplementary methods section.

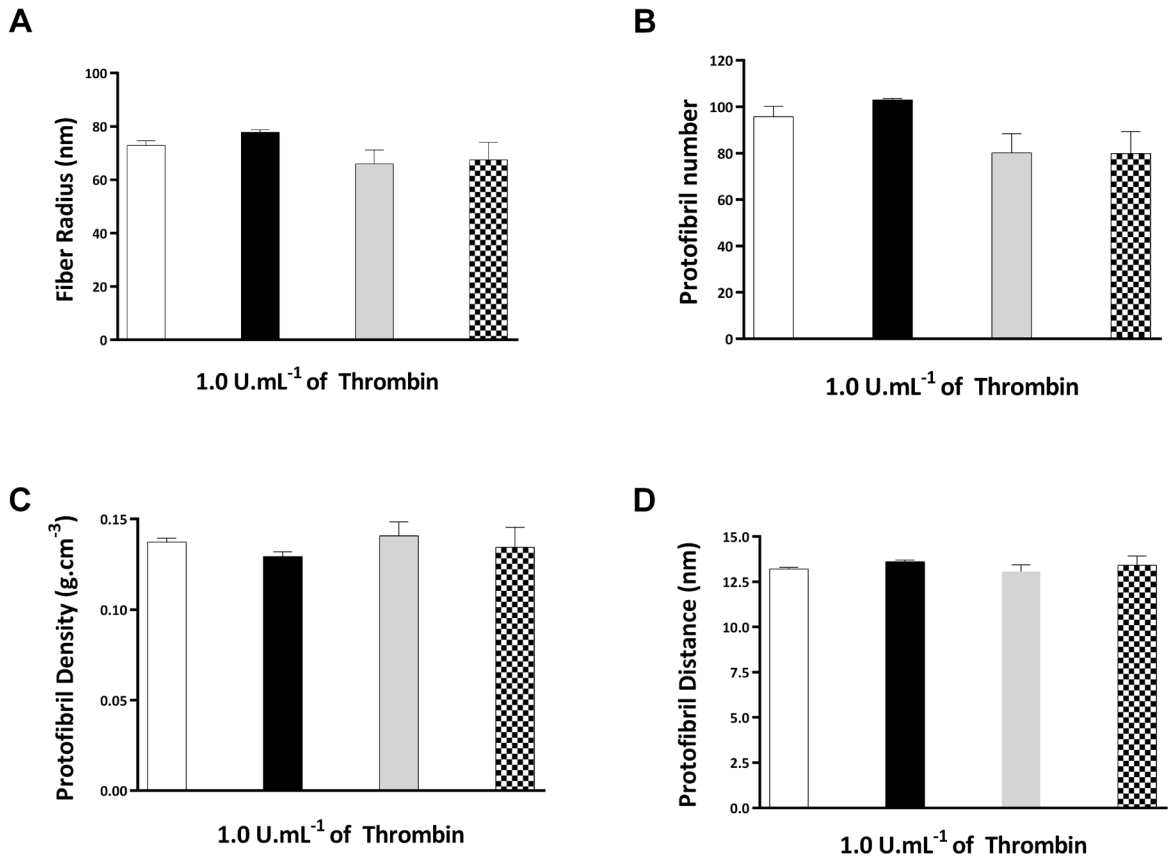

Figure S4 – No effect of γ' on the intrafibrillar structure of fibrin fibers by turbidimetry in plasma at 1.0 U.mL^{-1} of thrombin. Fibrinogen deficient plasma was diluted 1/10 and supplemented with 0.3 mg.mL^{-1} of purified γA/γA (white bars), γA/γ' (black bars), γA/γA:γA/γ' 60%:40% (grey bars) and γA/γA:γA/γ' 91%:9% (square patterned bars) fibrinogen, 10 mM $CaCl_2$ and thrombin concentration 1.0 U.mL^{-1}. A) Fibrin fiber radius; B) Number of protofibrils within fibrin fibers; C) Protein density of fibrin fibers; D) Distance between protofibrils inside the fibrin fibers. The results represent the mean values ± SD, n=3. Statistical significance, using a one-way ANOVA, are denoted with *p<0.05 for comparison between γA/γA and the other fibrinogen systems.

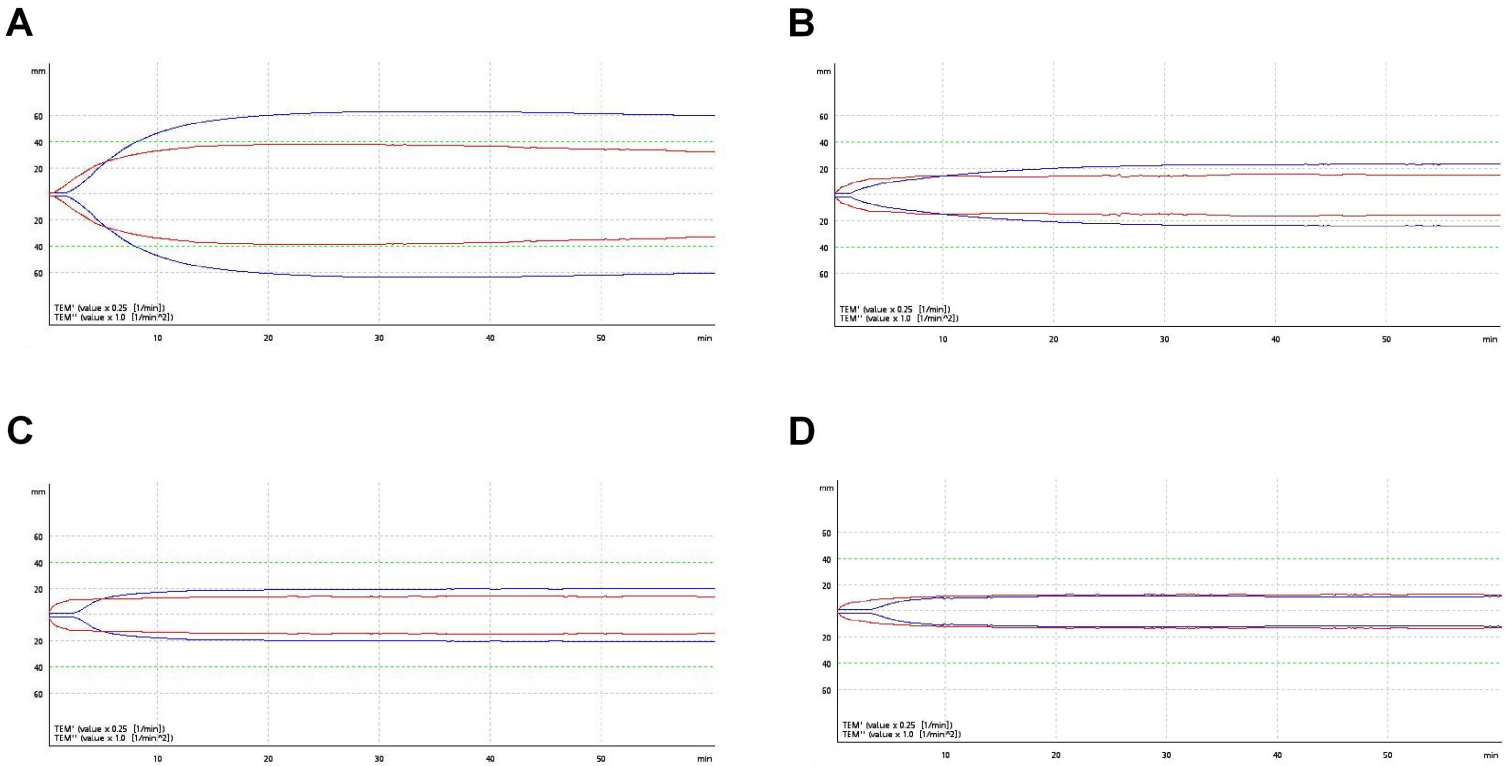

Figure S5 – Effects of thrombin and γA/γ' fibrinogen on clot stiffness by thromboelastometry assays. Thromboelastometric profile of A) whole blood, B) normal pooled plasma, C) fibrinogen deficient plasma supplemented with γA/γA fibrinogen, and D) fibrinogen deficient plasma supplemented with γA/γ' fibrinogen, clotted with 1.0 (blue) and 10 $U.mL^{-1}$ (red) thrombin and 10 mM $CaCl_2$. Fibrinogen deficient plasma was supplemented with 3 $mg.mL^{-1}$ of purified γA/γA and γA/γ'. The profiles shown are from one sample at each condition that are a close representation of the triplicate profiles measured.

Movie: Movement of the paramagnetic bead in a fibrin clot produced with 5 U.mL^{-1} thrombin using magnetic tweezers. The clot was produced with 0.5 mg.mL^{-1} fibrinogen, 2.5 mM $CaCl_2$ and human α-thrombin at final concentration of 5.0 U.mL^{-1}. The clot was immediately transferred to a capillary tube and placed between the electromagnets. The magnetic bead moves towards the applied magnetic field with an elastic component (small initial displacement). The viscous component is small for this clot, while the elastic recovery of the fibrin trapping the bead is apparent.

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